EFFECT OF DRY HEAT ON COLLAGEN AND LEATHER*

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ABSTRACT

Previous work relating to the effects of heating collagen and leathers dry or in the presence of limited amounts of water or other solvents is briefly reviewed.

The present investigation is primarily concerned with the chemical changes due to heating collagen dry. Moisture was removed by heating at 105° and the collagen then heated for varying periods of time at 140°, 150°, and 170°. Losses in weight, of nitrogen, and of amino acids were determined and changes in solubility examined.

At 140° and 150° losses of weight or nitrogen were small but there was an over-all loss of 20 percent in amino acid residues. At 170° losses were much greater, less than 50 percent of the original weight being recovered as amino acid residues after ten days of heating. Amino acids most affected were methionine, tyrosine, arginine, lysine, serine, and threonine. Although the general picture was one of oxidative degradation, heating in nitrogen instead of air only marginally decreased losses of amino acids.

A significant feature of the degradation was an increase in amide-nitrogen and keto acids. Similar increases occur on exposure to γ-radiation and it is suggested that a similar mechanism involving cleavage of carbon-nitrogen bonds may be involved.

The pH had little influence on degradation, and of the common tanning agents only the vegetable tannins appeared to cause any decrease in chemical breakdown.

The significance of the results in relation to deterioration under atmospheric conditions is briefly discussed.

*The work reported in this paper forms part of a research program sponsored by the U. S. Department of Agriculture under the authority of Public Law 480.

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INTRODUCTION

Present-day methods of shoe manufacture and stringent conditions in use impose ever-increasing demands on the resistance of collagen and leathers to heat. Much work has been done on the effect of moist heat (see, for example, the review by Seligsberger and Mann (1) and recent papers by Bowes and Raistrick (2–7) and Williams-Wynne (8)).

Relatively little, however, appears to be known about the effect of heat in the absence of water or at low moisture contents.

Two types of degradation may occur when collagen is heated: 1) chemical changes involving breakdown of the polypeptide chains and modification or loss of amino acids, and 2) physical changes associated with the loss of the characteristic helical structure. There is also evidence that the loss of water in itself leads to modification of the structure (9).

The physical changes associated with loss of helical structure lead to an over-all increase in volume, though with skin this is usually observed as a linear shrinkage. The temperature at which this loss of structure occurs depends on the nature and the amount of solvent present. As the moisture content is reduced, the shrinkage temperature increases (10, 11), and after drying over sulfuric acid neither collagen nor a variety of leathers (vegetable, chrome, alum, formaldehyde, and oil tanned) shrank on heating in paraffin to 155° [Bowes, unpublished].

Flory and Garrett (12) point out that the change undergone on heating has many of the characteristics of the fusion or melting of the crystalline regions in orientated polymers, water or other solvent acting as a diluent and depressing the melting point. Using a dilatometric technique, they showed that, with ethylene glycol as the diluent, the melting point rose from 42° to 118° as the volume ratio of collagen to solvent was raised from 0.08 to a level approaching 0.9. On extrapolating back to zero, this gave a melting temperature for the "pure" polymer of 145°. Water was reported to have an additional solvent effect, compared with ethylene glycol. Witnauer and Fee (11) have examined the effect of diluent on the shrinkage temperature by heating leathers containing various amounts of water, ethylene glycol, phenol, and formamide in mercury. Extrapolation to zero concentration indicated a limiting value of about 145° for collagen, 120° for vegetable-tanned leathers, and above 170° for formaldehyde-tanned leather. The lower value for the vegetable-tanned leather was attributed to the diluent effect of the tan itself.

More recently, differential thermal analysis (DTA) studies on dry collagen indicate an endothermic change at 230–240° (13 and unpublished work‡). Gelatin (14, 15) shows a similar endothermic change, suggesting it may not be related to loss of helical structure, and other proteins show a peak in the same range (16, 17). Morita (16) considers that peaks above 200° are related to

‡Private communication from C. H. Pearson, Dept. of Textile Industries, University of Bradford, Yorkshire, England, and unpublished work by J. E. Taylor.

chemical degradation. In support of this view, Kasarda and Black (18), using mass spectroscopy to follow thermal degradation, report that ammonia begins to be released at 130–150° and reaches a peak rate at 230°. This is followed by carbon dioxide with a peak at 280° and then by sulfur dioxide and hydrogen sulfide from those proteins containing sulfur.

The only study of the chemical degradation of collagen is that undertaken by Cassel (19), who examined the effect of heating in the dry state in vacuum, air, and oxygen at 150° and 170° and in oxygen at 125°. Damage as assessed by appearance and solubility increased with temperature and was least in vacuum and greatest in oxygen. Even the most degraded samples, however, still retained their fibrous appearance until placed in water, when up to 25 percent dissolved. Losses of amino acids as determined by paper chromatography were extensive, methionine, serine, tyrosine, lysine, hydroxylysine, phenylalanine, and valine, in that order, being the most affected. The results as a whole were indicative of oxidative degradation. It is also possible that some degree of crosslinking occurs on heating. The solubility of gelatin decreases on heating (20, 21) and recently Yannas and Tobolsky (22) have produced evidence suggesting the condensation of amino and carboxyl groups to give amide bonds.

It would seem from the above data that the maximum temperature to which dry collagen can be heated for short periods without physical changes is somewhere in the region of 220–250°. Chemical degradation, however, appears to start at lower temperatures, and, if heating is prolonged, may lead to the occurrence of these physical changes at lower temperatures.

The present investigation is primarily concerned with the extension of the studies made by Cassel (19) with the object of obtaining further information on the mechanisms by which breakdown occurs. From such knowledge it is hoped that ways by which the resistance of collagen and leathers can be increased may be indicated. Three temperatures of heating were chosen, 140° and 150° (as near the maximum that collagen will withstand for prolonged periods without chemical breakdown extensive enough to induce physical changes associated with loss of helical structure) and 170° (as a temperature at which appreciable breakdown was likely to occur, so facilitating the identification of the products).

EXPERIMENTAL

Raw Material

Collagen

The middle split of a commercially limed steerhide butt was delimed to pH 4.0 with acetic acid, washed thoroughly, and dehydrated with acetone. This material is referred to throughout as collagen. The sheepskin collagen included in the 150° and 170° series was prepared from commercial pickled and degreased hair sheepskins. These were delimed to pH 5.0 with sodium acetate, washed, and acetone dehydrated.

Tanned Collagens

The oxhide collagen was ground to pass 20 mesh and 20 g. was tanned by each of the following procedures.

a) Vegetable Tannage — The collagen was shaken with 400 ml. mimosa or myrobalans liquor (specific gravity 1.05) for three hours; a further 200 ml. of more concentrated liquor (sp. gr. 1.20) was then added and shaking continued for a further three hours. After leaving stationary overnight, the pH was adjusted to 4.0, shaking continued for three hours, and the tanned collagen washed and air dried.

b) Chrome Tannage — The sample was shaken in 400 ml. ten percent NaCl containing one percent H_2SO_4 for three hours; the final pH was 1.5. Six percent (w/v) of 50 percent basic commercial chrome tanning powder (25 percent Cr_2O_3) was added in two lots. The next day the pH was brought to 4.5 by the addition of eight percent $NaHCO_3$ solution. The tanned powder was washed in tap water and air dried. A further sample was treated similarly, using 24 percent (w/v) of the chrome tanning powder to give a higher level of tannage.

Final chrome contents expressed as Cr_2O_3 on air dry weight were: low level, 1.1 percent; high level, 7.2 percent.

c) Glutaraldehyde Tannage — The powder was shaken in 400 ml. ten percent NaCl and 2 g. glutaraldehyde added (eight ml. 25 percent solution). After one hour's shaking, the pH was brought to 7.5 with $NaHCO_3$ and shaking was continued for six hours.

The two commercial leathers were fatliquored but not dyed.

General Procedure

The following series of experiments were carried out:

1) collagen heated at 170° in air for one, three, five, ten, and 20 days;
2) collagen heated at 150° in air for one, three, five, and ten days, and in nitrogen for ten days;
3) collagen heated at 140° in air for five, ten, 15, 20, 30, and 40 days;
4) collagen adjusted to different pH values and heated at 150° in air for ten days;
5) tanned collagens heated at 150°C. in air for ten days;
6) commercial full chrome and chrome retan leathers heated in air at 170° for one, three, five, and ten days.

All samples were heated on the middle shelf of an electric oven, 46 x 46 x 36 cm., with an air vent, first for 24 hours at 105° to remove moisture and then at the prescribed temperature. For the sake of convenience, several one, 0.5, and 0.2 g. samples were heated separately, so that whole samples could be taken for the different determinations and results calculated back directly to moisture-free weight or total nitrogen of the original material. Bulk samples spread thinly on aluminum trays were also included to provide material for additional tests.

Analytical Methods

Amino Acid Analysis

Samples were hydrolyzed in 6 N HCl at 105° for 20 hrs. and amino acids separated and determined by the procedure of Hannig (23), using a Bender and Hobein automatic analyzer. Basic amino acids were separated on a 20 x 1 cm. column of IR 120, 20–25 mμ at 57°, using 0.38 M citrate buffer, pH 4.46, for elution. Hydroxyproline was determined directly on separate samples by an automated Stegemann procedure (24).

Total Nitrogen

Samples were digested by macro- or micro-digestion procedures, using a copper-selenium catalyst, and nitrogen was determined by an automated colorimetric method, using an alkaline-phenol-hypochlorite reagent (24).

Amide-Nitrogen and Free Ammonia

Amide-N plus free ammonia was determined by hydrolysis under reflux with 20 ml. 2 N HCl for one hr., followed by steam distillation using a Markham Still (25). Hydrolysates were neutralized to pH 5.0 with 4 N HCl and aliquots distilled from saturated borate-NaOH buffer, pH 11.5. Distillates were collected in one percent boric acid and titrated with 0.01 N HCl. For free NH_3, samples (0.25 g.) were shaken with 20 ml. distilled water for one hr., the solution filtered and made up to 50 ml., and aliquots distilled from borate buffer as before.

Aldehydes

Samples were brought into solution by hydrolyzing in 6 N HCl at 105° for 20 hrs. or by heating in water at 100°. Aldehydes were determined in the filtrates by reaction with *N*-methylbenzothiazolone hydrazone (MBTH) (26).

Keto Acids

After some preliminary tests, hydrolysis with 4 N HCl for 16 hours at 105° was decided upon (see Bowes and Raistrick (7)). Keto acids were then determined by the method of Lappin and Clarke (27).

N-Terminal Residues

The samples (1–2 g.) were reacted with 2, 4-dinitrofluorobenzene (FDNB) in saturated $NaHCO_3$ solution in the dark. After extraction with diethyl ether to remove excess reagent, both solution and residue were hydrolyzed in 6 N HCl

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RESULTS

I. Collagen

Changes in Appearance and Feel

Heating for one day at 150° had relatively little effect, but after long periods of heating the samples became progressively more yellow in color and the skin pieces began to lose area and to become brittle. At 170° damage was observed after one day, and after ten days samples were dark brown in color and broke up on handling.

Some loss in area (11 percent) resulted from heating at 105° to expel moisture. Further losses occurred on heating; there was a total decrease in area of 28 percent after ten days at 150° and of 44 percent after the same time at 170°. All the heated samples shrank on being placed in water at 22°, indicating extensive loss of structural stability.

Losses in Weight and of Nitrogen

Losses in weight over and above that due to removal of moisture at 105° were relatively small at 140° and 150° (Fig. 1) but became as high as 23 percent after 20 days at 170°, indicating extensive breakdown of protein. Losses of nitrogen were negligible at 140° for periods up to 20 days, and also at 150° in nitrogen for ten days (Fig. 2). In air, losses at 150° were only three percent after ten days but at 170° losses were up to 15 percent. In this series there was some indication of specific loss of nitrogen in the early stages; as much as one quarter of the weight loss after one day was attributable to nitrogen only. This nitrogen could arise from breakdown of amide groups or guanidyl groups of arginine. As heating was continued, the ratio of nitrogen to weight loss became more compatible with general over-all degradation of amino acids.

Solubility

In an attempt to assess degradation, samples were extracted with water or 0.5 M acetic acid at 20° and 100°, and the nitrogen in the extracts was determined. Preliminary tests showed that, using one-gram samples, essentially all the soluble nitrogen was removed with one extraction with 40 ml. for 24 hrs., and this was adopted as the standard procedure.

Rather surprisingly, solubility in acetic acid was not appreciably greater than in water. The heated samples showed little tendency to swell in the acid, sug-

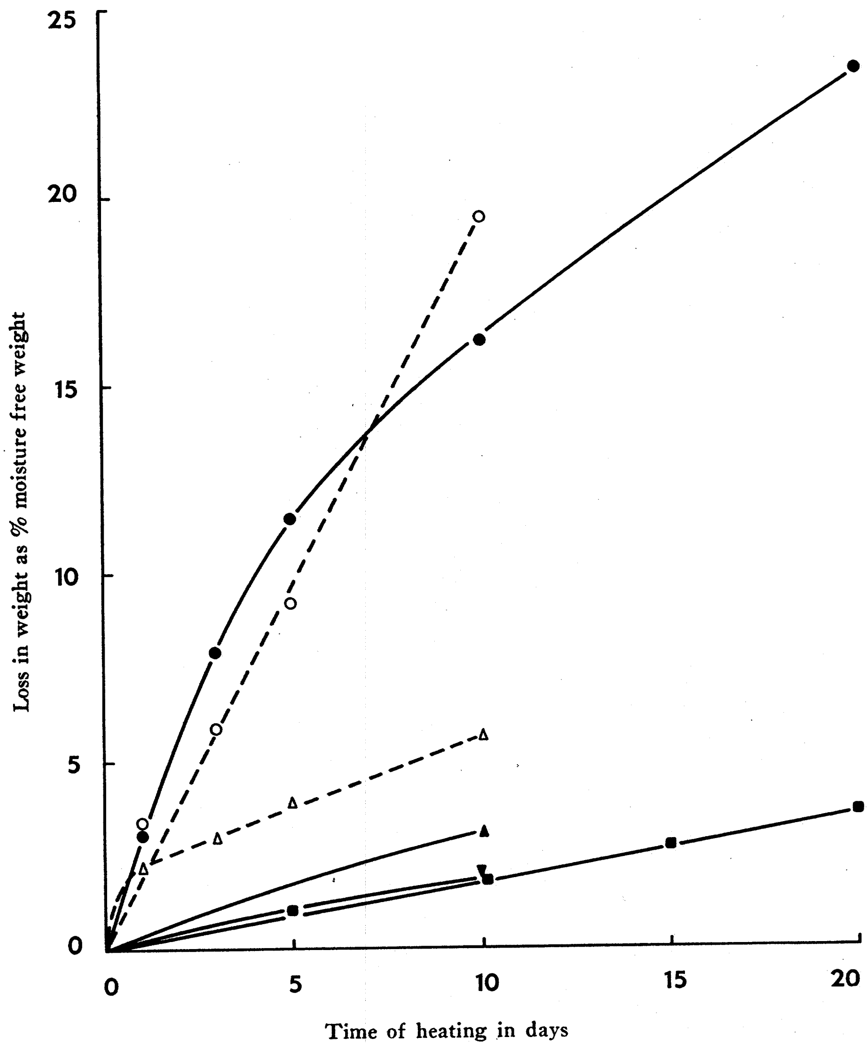

FIGURE 1.—Loss in weight on heating at 140°, 150°, and 170°.
■ 140° in air
▲ 150° in air
● 170° in air
▼ 150° in N_2
Solid symbols — steerhide collagen
Open symbols and broken lines — sheepskin collagen

gesting that the insoluble fraction may have become crosslinked in some way (20–22).

Nitrogen extractable at 20° increased sharply during the early stages of heating and then began to level off, and in the case of the steerhide collagen actually

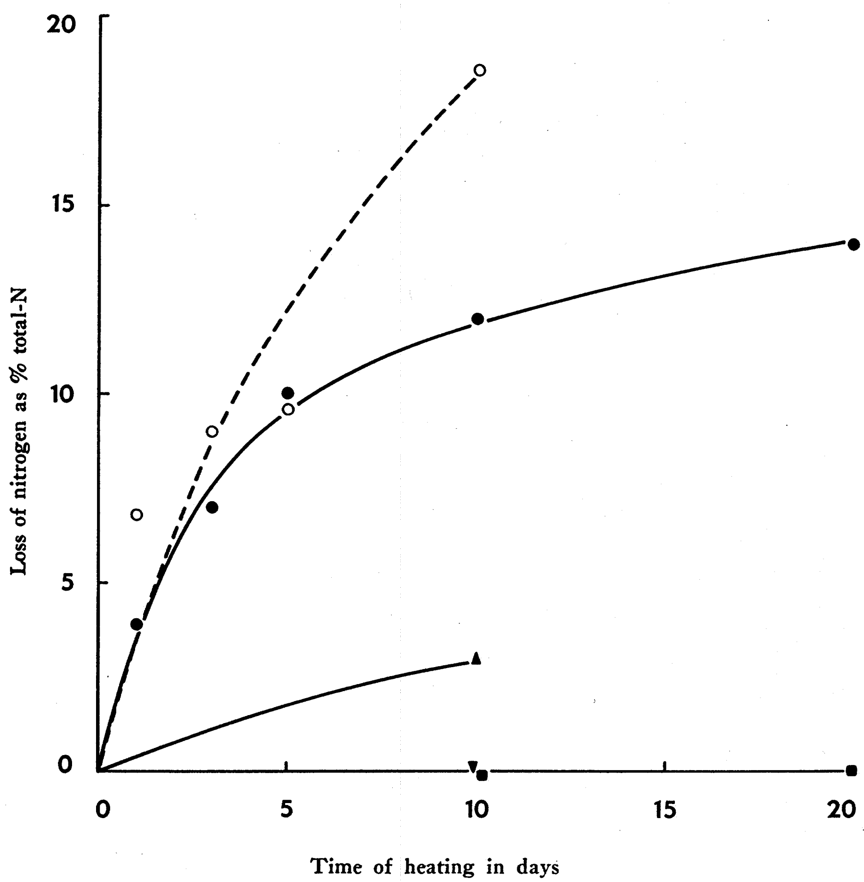

FIGURE 2.—Loss of nitrogen on heating at 140°, 150°, and 170°.
■ 140° in air
▲ 150° in air
● 170° in air
▼ 150° in N_2
Solid symbols — steerhide collagen
Open symbols and broken lines — sheepskin collagen

to decrease (Fig. 3). After 20 days of heating (not shown in the figure) the soluble nitrogen was reduced to 11 percent. This may be ascribed to further breakdown and loss as volatile products as the heating is prolonged. With the sheepskin collagen, extraction at 100° dissolved between 90 and 95 percent of the total nitrogen, but with steerhide only 70–75 percent was extracted.

All extracts absorbed in the ultraviolet range with indication of a peak at 270 nm. (Fig. 4). Absorbance of the 20° extracts was about one third to one half of that of the corresponding 100° extract, but was higher in relation to their nitrogen content. Compounds responsible for the increased U.V. absorbance are

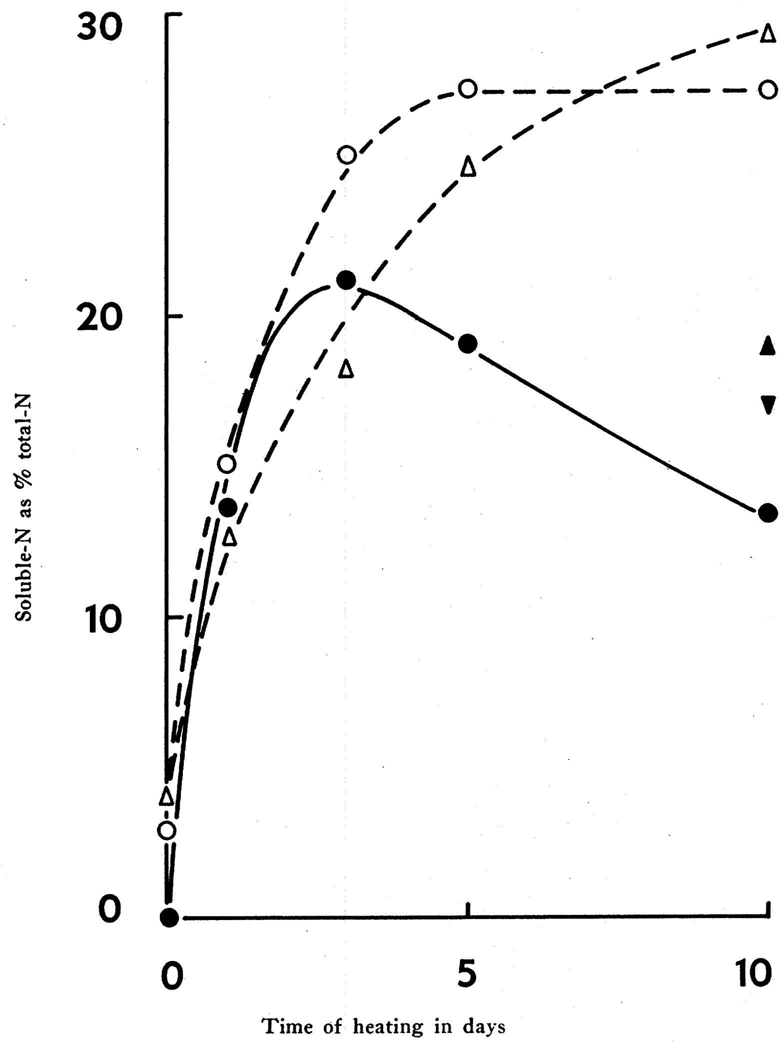

FIGURE 3.—Solubility in water at 20° — Nitrogen dissolved as percent total Nitrogen of original sample.

▲ 150° in air
● 170° in air
▼ 150° in N_2
Solid symbols — steerhide collagen
Open symbols and broken lines — sheepskin collagen

therefore not, as might have been expected, primarily located in the high molecular weight insoluble residue. U.V. absorbance of hydrolysates was lower than that of corresponding 100° extracts, suggesting that some aggregation occurs during hydrolysis, leading to the concentration of the U.V. absorbing components in one to three percent of insoluble residue.

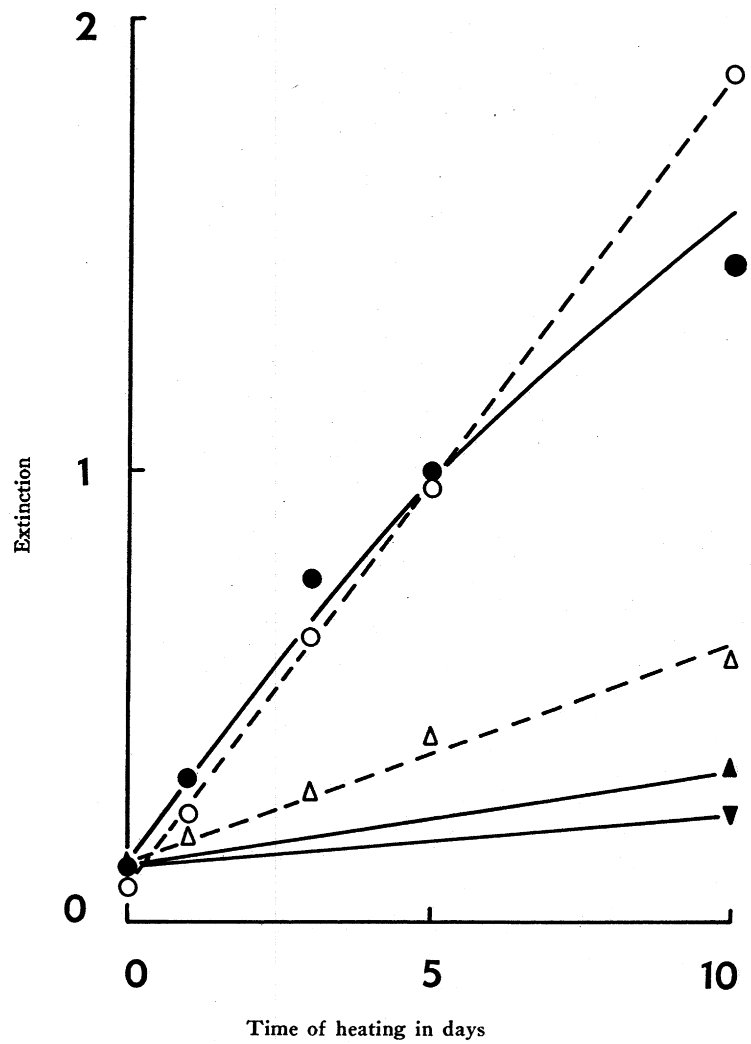

FIGURE 4.—Ultraviolet absorption of 100° aqueous extracts. Extinction at 270 nm. in 1-cm. cells at concentrations equivalent to 0.4 mg. original air dry material per ml.

▲ 150° in air
● 170° in air
▼ 150° in N_2
Solid symbols — steerhide collagen
Open symbols and broken lines — sheepskin collagen

Amino Acid Analyses

Amino acid analyses were carried out on the steerhide collagen heated at 170° for various times and at 140° and 150° for ten days. No significant losses occurred during the heating at 105° to expel moisture.

TABLE I

AMINO ACID COMPOSITION OF STEERHIDE COLLAGEN HEATED IN AIR AT 170° FOR VARIOUS TIMES

(Results expressed as g.-residues recovered per 100 g. moisture-free original collagen)

	Control	Days Heated 1	3	5	10	20
Hydroxyproline	12.04	10.77	5.39	6.11	5.28	4.80*
Aspartic acid	5.28	4.73	4.67	3.88	3.77	3.37
Threonine	1.67	1.14	0.79	0.45	0.28	nil
Serine	2.74	1.63	1.01	0.42	0.24	nil
Glutamic acid	10.09	8.94	7.15	4.70	5.22	3.68
Proline	11.15	10.51	8.61	6.24	4.48	1.93
Glycine	19.32	16.98	16.20	12.85	11.34	8.17
Alanine	8.10	7.71	6.42	5.56	5.16	3.52
Valine	3.70	2.08	2.09	1.51	1.38	0.90
Methionine	0.75	0.28	trace	trace	nil	nil
Iso-leucine	1.51	1.21	1.02	0.82	0.64	0.34
Leucine	2.77	2.42	2.08	1.60	1.44	0.75
Tyrosine	0.53	0.21	0.27	trace	trace	trace
Phenylalanine	1.88	1.77	1.51	1.18	1.19	0.71
Histidine	0.45	0.22	0.16	0.20	0.23	trace
Lysine	3.82	2.25	1.61	1.15	1.12	0.74
Hydroxylysine	0.72	0.49	1.10	1.25	0.49	0.39
Ornithine	0.11	0.36			0.78	0.64
Arginine	8.11	5.66	2.86	1.61	1.18	0.76
Unknown† 1			1.43	0.91	0.96	0.82
Unknown 2						0.20
Unknown 3					trace	0.21
NH_3	(0.53)	(1.19)	(1.99)	(2.07)	(2.27)	(2.46)
Total residues recovered	94.7	79.4	64.4	50.4	45.2	31.9
% Nitrogen in hydrolysate recovered	102.5	84.4	83.8	69.1	66.1	62.5

*Estimated from ninhydrin color yield and general trend of losses.

†Unknowns were calculated on the basis of the color yields of cystine (1) and lysine (2 and 3) and an average residue weight of 100.

Consider first the 170° series (Table I). Appreciable losses of most amino acids occurred after heating for only one day, in particular of methionine, tyrosine, arginine, lysine, serine, and histidine, in that order. Essentially all the methionine and tyrosine were lost after three to five days of heating, and with serine and threonine there was a steady decrease with time of heating to almost zero at five days. Losses of arginine and lysine were high during the early stages and then leveled off. Aspartic acid, phenylalanine, alanine, and glycine were apparently the least affected. Ornithine was detected in increasing amounts, presumably derived from arginine. However, this represents only a small proportion of the arginine lost, perhaps because the arginine also breaks down in the same way as lysine. Small amounts of three new ninhydrin positive compounds were observed after five days, one running in the same position as cystine, another just after ammonia, and a third after phenylalanine on both columns.

Losses at 140° and 150° were similar and much less than at 170° (Table II). With most amino acids, losses after ten days were comparable with those occurring in one day at 170°. Aspartic acid, proline, and phenylalanine appear to be more sensitive to rise in temperature than most of the amino acids, and glycine, valine, lysine, and arginine less so. There was no evidence that heating in nitrogen as opposed to air had any significant effect on losses of amino acids.

As the time of heating was extended, the percentage of the original weight recovered as amino acid residues decreased (Table III) and after 10–20 days of heating at 170° about 40 percent of the original weight remained unaccounted for as either volatile material or amino acid residues. The total nitrogen recovered as amino acids and ammonia was also low, leaving 15–43 percent, depending on the time and temperature, of the original nitrogen of the sample to be classified as non-amino nitrogen. Most of this unaccounted-for nitrogen was returned in Kjeldahl nitrogen determinations on the hydrolysates.

There was little loss in weight or of nitrogen on heating at 140° and 150° for ten days but again nearly 20 percent of the weight and about 15 percent of the nitrogen was not recovered as amino acids. This indicates the presence of a high proportion of material in the heated residues which is crosslinked through bonds involving nitrogen and stable to acid hydrolysis.

Aldehydes and Keto Acids

Aldehydes and keto acids were considered to be possible breakdown products of the amino acids. The choice of a suitable procedure for bringing the collagen into solution was a problem in both these determinations, since hydrolysis is likely to lead to losses or the formation of artifacts. In preliminary experiments, 8 M urea and 2 M calcium chloride at room temperature and enzymes such as collagenase and pronase were tried but did not prove very satisfactory. Finally aldehyde determinations were made on the aqueous 100° extracts which dissolved some 90–95 percent of the sheepskin collagen and 70–75 percent of the steerhide

TABLE II

LOSSES OF AMINO ACIDS ON HEATING FOR TEN DAYS AT 140°, 150°, AND 170°

	G.-Residues Recovered — % Original Wt.				Percentage Losses			
	170° In Air	150° In Air	150° In N_2	140° In Air	170° In Air	150° In Air	150° In N_2	140° In Air
Hydroxyproline	5.28	10.61	10.93	11.48	56	12	9	5
Aspartic acid	3.77	5.22	4.92	4.61	29	1	7	13
Threonine	0.28	1.41	1.43	1.23	83	16	14	26
Serine	0.24	1.92	2.18	1.86	91	30	20	32
Glutamic acid	5.22	8.52	8.23	7.79	48	16	18	23
Proline	4.48	10.55	11.01	11.07	60	5	1	1
Glycine	11.34	14.91	17.82	16.53	41	23	8	14
Alanine	5.16	7.36	6.65	6.61	36	9	18	18
Valine	1.38	2.17	2.30	2.06	63	41	38	44
Methionine	0	0.48	0.42	0.55	100	36	44	27
Iso-leucine	0.64	1.34	1.20	1.21	58	11	20	20
Leucine	1.44	2.55	2.88	2.54	48	8	(4)	8
Tyrosine	trace	0.44	0.22	0.33	100	24	58	38
Phenylalanine	1.19	1.99	1.73	1.79	37	(6)	8	5
Histidine*	0.23	0.84	0.70	0.72	49	(87)	(56)	(60)
Lysine	1.12	1.80	1.87	2.08	71	53	51	46
Hydroxylysine*	0.49	} 1.11	} 0.82	} 0.49	32	—	—	—
Ornithine	0.78				(700)	—	—	—
Arginine	1.18	4.98	5.52	5.45	86	39	32	33
Unknowns	0.96	trace	not detected		—	—	—	—
Ammonia	2.27	1.88	1.25	0.98	(328)	(254)	(136)	(185)
Total residues recovered	45.2	78.2	80.8	78.4	50	17	15	17
% N in hydrolysate recovered	66.1	94.0	93.5	92.9	—	—	—	—

( ) indicates percentage gain.

*The amounts of these amino acids are small and hence susceptible to errors. There is also interference by unknown compounds which appear to be eluted in the same region

TABLE III

WEIGHT AND NITROGEN DISTRIBUTION OF HEATED COLLAGENS

	At 170° In Air (Days)						At 150° Air	At 150° N_2	At 140° Air
	0	1	3	5	10	20	10	10	10
Weight Distribution — % Original Weight									
Series I — Sheepskin Collagen									
Lost on heating	0	3	6	9	20				
Recovered as amino acid residues	90	75	62	56	40				
Unaccounted for	10	22	32	35	40				
Series II — Steerhide Collagen									
Lost on heating	0	3	8	11	16	23	3	2	1
Recovered as amino acid residues	95	79	64	50	45	32	78	81	78
Unaccounted for	5	18	28	39	39	45	19	17	21
Nitrogen Distribution — % Total-N of Original Material									
Series I — Sheepskin Collagen									
Lost on heating	0	7	9	10	19				
Recovered as amino acids or NH_3	100	84	74	66	51				
Unaccounted for	0	9	17	24	30				
Series II — Steerhide Collagen									
Lost on heating	0	4	7	10	12	14	3	0	0
Recovered as amino acids or NH_3	100	81	73	60	55	43	82	86	84
Unaccounted for	0	15	20	30	33	43	15	14	16

collagen, and keto acids were determined on partial hydrolysates (see Analytical Methods).

Spectrophotometric determinations of aldehydes after reaction with MBTH to give the azine were not very satisfactory, owing to interference from other products in the extract. However, by comparison of freshly made up reaction mixtures with those incubated for 24 hrs. at 40° to promote reaction with MBTH, it was possible to distinguish a definite peak at 315 nm and a smaller less well defined peak at 370 nm. Both saturated and unsaturated aldehydes, therefore, appear to be present. Both peaks increased with the time of heating of the collagen at 170°. The colorimetric determination involving further reaction with ferric chloride gave more satisfactory results, and small but definite increases in aldehyde groups were demonstrated (Table IV). These figures are in all probability too low, owing to incomplete solution of the collagen, and even if all free aldehyde groups are returned the values obtained do not necessarily represent all those formed during heating, since some interaction with amino or other groups may well have occurred.

Only a limited number of keto acid determinations were made. The results indicate a significant increase on heating at all three temperatures, but in terms of weight these increases represent less than two percent of the original material, only a small proportion of the material unaccounted for as amino acids.

Amide-Nitrogen and Free Ammonia

There were marked increases in the nitrogen returned as ammonia in the amino acid analysis of the heated collagens. The volatile nitrogen of extracts of the heated collagen indicated that little of this was present initially as ammonia, suggesting that the ammonia present in hydrolysates arose primarily from amide groups. Hydrolysis under mild conditions and steam distillation, as in the usual amide determination (25), confirmed this view (Table IV).

Amide-nitrogen plus free ammonia increased with time and temperature of heating, and after ten days was about three times greater at 170° than at 140°. Free ammonia, probably arising from breakdown of the amide groups during heating, formed only about ten percent of the total.

N-Terminal Residues

Determinations were complicated by the dark color of the hydrolysates and the presence of obvious artifacts. It was obvious, however, that values were low with all samples examined, increasing from 0.2 mmole per 100 g. in the control to 0.3 mmole per 100 g. in the sample heated at 170° for ten days.

Clearly, cleavage of peptide bonds to give amino groups, due either to traces of moisture in the early stages of heating or to other causes, is not a feature of the degradation.

TABLE IV

ALDEHYDES, KETO ACIDS, AND AMIDE-NITROGEN OF HEATED COLLAGENS

(mmoles per 100 g. dry heated collagen)

	Days Heated						
	0	1	3	5	10	20	40
*Aldehydes**							
Sheepskin collagen 170° in air	0.6	1.2	2.4	3.4	4.0	—	—
Steerhide collagen 170° in air	0.3	1.4	3.6	3.6	3.5	—	—
Keto Acids†							
Steerhide collagen 170° in air	3				26		
Steerhide collagen 150° in air	3				17		
Steerhide collagen 150° in N_2	3				17		
Steerhide collagen 140° in air	3				17	23	25
Amide-Nitrogen + Free Ammonia							
Sheepskin collagen 150° in air	25	39	47	63	75		
Steerhide collagen 170° in air					200		
Steerhide collagen 150° in air					79		
Steerhide collagen 150° in N_2					72		
Steerhide collagen 140° in air	35			49	60	81	91

*Colorimetric method of Sawicki *et al.* (26); glyceraldehyde as standard.
†Lappin and Clark (27); pyruvic acid as standard.

Effect of pH on Degradation

In a further series, collagen was adjusted to various pH values from 0.2 to 4.0, giving values for pH of water extract between 1.8 and 3.8, and heated at 150° and 170° for ten days (Table V). Increases in amide-N and keto acids were independent of pH, and losses of amino acids in the samples adjusted to pH 2.8 and 4.8 and heated at 150° for ten days were not significantly different. Thus, there is no evidence that the pH of the sample has any significant effect on the course of degradation.

II. Leathers

There was little evidence that either chrome or glutaraldehyde tannages led to any reduction in degradation due to heating at 150° for ten days (Table VI). Losses in weight were of the same order as with the untanned control sample and losses of nitrogen were in two cases greater. Increases in amide-nitrogen due to heating were less with the low chrome sample than with the control. However, the high nitrogen loss of this sample suggests that some breakdown of amide may have occurred. The sum of the nitrogen lost plus amide nitrogen is of the same order as the corresponding figures for the high chrome and glutaraldehyde-tanned samples (11.1–11.8) and higher than that of the control, 6.9 percent. The increases in solubility of these three samples are also greater than that of the control. The vegetable tannages appear to offer some degree of protection to heat degradation, although weight losses were greater, and losses of nitrogen and increases in solubility were less.

With the two commercial leathers, losses in area and weight were the same as those observed with collagen but losses of nitrogen during heating were less, especially with the chrome retan leather, an effect presumably associated with pH and the retention of more volatile products, such as ammonia, by the rather more acid type of leather (Table VII). Amide-nitrogen increased with time of heating as before. The combined figures for amide-nitrogen and loss of nitrogen are less with the chrome retan (16.7 percent) and the full chrome leather (22.5 percent) than with the untanned pelt (24.1 percent).

DISCUSSION

It has been shown that chemical degradation of collagen occurs rapidly at 170°, and after only one day there is extensive loss of weight, loss of nitrogen, and general loss of amino acids. As might be expected, there is little increase in α-amino groups, indicating only very limited hydrolytic chain scission due to traces of water. At 140 and 150° breakdown is much less but appears to be of a similar type. Changes occurring in ten days of heating roughly correspond to those occurring in one day at 170°.

Overall losses of amino acids increased steadily with time and temperature of heating but losses of individual amino acids were not always consistent. Methio-

TABLE V

EFFECT OF pH ON DEGRADATION

		pH			
	Controls	1.8	2.3	3.4	3.8
			Heated at 150° for 10 Days		
Loss in weight on heating (percent)	0	—	3	2	2
Loss of nitrogen	0	—	0	0	0
Weight recovery (as amino acid residues percent)	95	—	78	—	80
Nitrogen recovery (as amino acids and ammonia percent)	100	—	94	—	96
Amide-N + NH_3 (mmoles per 100 g.)	33	49	51	56	61
Keto acids (mmoles per 100 g.)	3	15	13	14	16
			Heated at 170° for 10 Days		
Amide-N + NH_3 (mmoles per 100 g.)	33	—	108	129	131
Keto acids (mmoles per 100 g.)	3	—	29	—	29

TABLE VI

EFFECT OF HEAT ON TANNED STEERHIDE COLLAGEN

Tannage	Untanned		Mimosa		Myrobs.		Low Chrome		High Chrome		Glutaraldehyde	
Days heated at 150°	0	10	0	10	0	10	0	10	0	10	0	10
Loss in weight (as % m.f.wt.)	—	3	—	8	—	8	—	3	—	4	—	4
Loss of N (as % Total-N)	—	3	—	0	—	0	—	7.7	—	1	—	5
Amide-N (as % Total-N)	2.4	6.4	2.1	6.1	1.5	2.4	0	3.4	1.8	11.3	2.4	6.8
Solubility — N extracted at 20° (as % Total-N)	0.3	18	0	4	0	1.6	0	11.6	1.1	14	0	27

TABLE VII

EFFECT OF HEAT ON COMMERCIAL CHROME AND CHROME RETAN LEATHERS

Tannage	Acetone Dehydrated					Full Chrome					Chrome Retan				
Days heated at 170°	0	1	3	5	10	0	1	3	5	10	0	1	3	5	10
Loss in wt. (as % m.f.wt.)	0	3	8	11	16	0	5	7	13	18	0	4	7	10	16
Loss of N (as % total-N)	0	4	7	10	12	—	4	—	7	9	0	0	0	0	0
Loss in area (as % of air dry)	0	24	27	31	44	0	24	27	40	44	0	24	31	42	43
Amide-N (as % total-N)	2.1	3.9	7.0	9.7	12.1	6.2	6.8	8.9	10.4	13.5	2.8	4.8	8.0	11.3	16.7

nine and those containing basic or hydroxy groups tended to be most affected but rather surprisingly aspartic acid, generally considered to be rather labile, was one of the least affected. Losses of arginine and lysine at 170° were rapid at first, followed by a general leveling off. This, together with the high proportion of nitrogen also lost during the early stages, is indicative of specific loss from the basic side chain groups, not necessarily involving main chain scission.

Although replacement of air by nitrogen had apparently very little influence on the course of breakdown, the general picture is suggestive of oxidative degradation leading to loss of amino acids and production of ammonia and carbon dioxide. The degradation in many ways resembles that due to γ-irradiation, and the increase in amide-nitrogen and keto acids suggests that a similar mechanism may be involved

$$\text{—}\underset{\substack{|\\ R_1}}{CH}\text{—CO—NH—}\underset{\substack{|\\ R_2}}{CH}\text{—CO—} \rightarrow \text{—}\underset{\substack{|\\ R_1}}{CH}\text{—CO—}NH_2 + O{=}\underset{\substack{|\\ R_2}}{C}\text{—CO—}$$

(see, for example, Jayko and Garrison (29), Garrison and Weeks (30), and Bowes and Moss (31)). As with γ-irradiation, increases in amide-nitrogen are greatly in excess of keto groups, presumably because the latter are more labile or because they interact with other breakdown products. The presence in the heated residues of a high proportion of material unaccounted for in terms of amino acid residues is suggestive of such interaction. With the collagen heated at 170° for ten days, as much as 50 percent of the weight and 40 percent of the nitrogen in the heated residue remains unaccounted for. Interaction of keto acids with guanidyl groups (32) or of aldehydes with amino groups are possibilities. Compounds so formed might well contain several amino acid residues and be difficult to detect, owing to their low ninhydrin color yield or because their relatively large molecular size causes them to be retained at the top of the chromatographic columns.

The evidence at this stage, therefore, suggests that degradation by dry heat involves first specific loss of labile side chain groups, *e.g.,* guanidyl, followed by chain scission to give amide and keto groups. This is then followed by further breakdown and loss of amino acids and also interaction between degradation products to give compounds containing nitrogen not recoverable as amino acid nitrogen on hydrolysis. Breakdown in even one day at 170° is apparently sufficient to reduce the stability of the helix to a level which allows shrinkage to occur in water at room temperature.

Production of ammonia and increase in amide-nitrogen appears to be the most sensitive indication of the start of chemical damage. This also appears to be true of other proteins, thermal degradation studies using mass spectrometry indicating that the first product is ammonia, which begins to increase above background between 130–150° and reaches a peak at 230° (18).

The maximum temperature to which collagen can be heated for long periods, therefore, probably lies around 140°, although good resistance for relatively short periods is possible at higher temperatures. The increases in amide-nitrogen occurring at this temperature may not appear to be very great but it should be realized that an increase of even ten moles per 100 g. is roughly equivalent to the loss of one out of every hundred amino acids, and if chain scission is involved a decrease in chain molecular weight from 100,000 to 10,000.

Although replacement of air by nitrogen only slightly reduced increases in amide-nitrogen, it is still possible that the incorporation of antioxidants might extend the permissible times and temperatures of heating.

Tanning agents have been found to have relatively little effect in retarding degradation, except possibly the vegetable tans, especially myrobalans. Such effects may be associated with the antioxidant properties of their constituents.

The present findings may have some relevance to the deterioration of leather due to prolonged exposure at lower temperatures, *e.g.*, to bookbinding and upholstery leathers (33, 34). Increase in ammonia and amide-nitrogen is also characteristic of this type of deterioration and a similar mechanism could be involved. Deterioration of this kind is, however, associated with low pH values due to absorption of acid, and so far there is no evidence that degradation by dry heat is favored by low pH values, so here the analogy fails.

ACKNOWLEDGMENTS

The authors thank Mrs. A. Dexter, Mrs. C. F. Davies, Mr. P. Tamulevicius, and Miss M. Burt for skilled technical assistance.

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